

AMENDMENTS TO THE SPECIFICATION:

Page 2, lines 1-12 please amend as follows:

extensively studied. The beta-casomorphin with the sequence Tyr-Pro-Phe-Pro-Gly-Pro-Ile (SEQ ID NO:15) is the principal opioid peptide in bovine milk and is called BCM-7 (beta-casomorphin (1-7); Chang *et al.* (1985) Journal of Biological Chemistry, 260, 9706-9712). Apart from this BCM-7 fragment at amino acid positions 60-66 of the beta-casein molecule, smaller fragments of BCM-7 like Tyr-Pro-Phe-Pro (beta-casomorphin (1-4)) (SEQ ID NO:16) and Tyr-Pro-Phe-Pro-Gly (beta-casomorphin (1-5)) (SEQ ID NO:17) at amino acid positions 60-63 and 60-64 respectively as well as all larger BCM-7 related peptides up to a chain length of 11 amino acids (at amino acid positions 60-70) display at least some degree of opioid activity. The N-terminal tripeptide of BCM-7, i.e. the sequence Tyr-Pro-Phe at position 60-62, has no opioid activity. A genetic beta-casein variant called A1 (having a histidine rather than the proline residue of A2 beta-casein at amino acid position 67) is claimed to lead to the formation of increased levels of the BCM-7 molecule.

Page 10, lines 8-21 please amend as follows:

Peptides or polypeptides having four to forty amino acid residues that are not hydrolysable by subtilisin (EC3.4.21.62), preferably subtilisin Carlsberg are understood to be peptides or polypeptides that after an incubation of 2 hours at pH 8.0 and 60 degrees C in a suspension or solution containing 20 g/l protein and an enzyme to

substrate ratio of 0.12 AU-A (Anson Units Alcalase) Protease Units per gram protein remain intact. The AU-A Protease Unit is defined as specified in the Analytical Method LUNA# 2003-32153-01 as issued by Novozymes (Denmark). Intact meaning that after the incubation the original peptide or polypeptide forms more than 80 %, preferably more than 90 %, more preferably more than 95 % of the resulting enzyme reaction products. In practice the enzyme digestion is carried out under the conditions indicated and using 40 microliter of Alcalase per gram of substrate protein present. Examples of such a non-hydrolysable peptide is the VYFPFGPIPN (SEQ ID NO:1) peptide resulting from the beta-casein hydrolysis described in Example 4. Another example of such a non-hydrolysable polypeptide is the 33-mer described in Example 6.

Page 14, lines 1-25, please amend as follows:

Software for performing BLAST analyses is publicly available on the internet through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pair (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold. These initial neighbourhood word hits act as seeds for initiating searches to find HSPs containing them. The word hits are extended in both directions along each sequence for as far as the, cumulative alignment score can be increased. Extensions for the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score

goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

Page 16, lines please amend as follows:

Preferably at least 80% of the toxic proline rich peptides or polypeptides which are formed upon an incubation of peptides or polypeptides or protein with subtilisin (EC 3.4.21.62) preferably a *Bacillus licheniformis* subtilisin (or subtilisin Carlsberg) under neutral pH conditions are hydrolysed by the proline specific endoprotease according to the invention. The formation of such subtilisin resistant peptides is illustrated in Example 4 of this application. Such subtilisin resistant proline rich peptides are often related with the diseases mentioned above. Examples of these peptides are BCM-7, BCM-7 related peptides i.e. peptides comprising the amino acid sequence YPFP (SEQ ID NO:2) as present at position 60 to 63 of the beta-casein molecule. Furthermore gliadin derived peptides comprising the motif Gln-Xxx-Pro (Q-X-P), for example the PYPQPQLPY epitope (SEQ ID NO:5) as well as other subtilisin resistant molecules comprising this Q-X-P or E-X-P motif that can be obtained from gliadin hordein, secalin or avenin are examples hereof. More preferably at least 90%, still more preferably at least 95% and most preferably at least 99% of proline rich peptides which would be

formed by hydrolysis by subtilisin are broken down or are not formed by using the proline specific endoprotease according to the invention. Most preferably these proline rich peptides can be degraded according to the process of the invention under conditions below pH 5.5.

Page 23, lines 22-34, please amend as follows:

It is well known that peptide bonds involving negatively charged residues such as Glu (E) and Asp (D) form poor substrates for proteases. Also the natural gastrointestinal proteolytic enzymes cannot cope with these residues as evidenced by the isolation of the gastric and pancreatic protease resistant peptide WQIPEQSR (SEQ ID NO:6) from gliadin (cf. Shan *et al*). The latter publication also makes clear that the ubiquitously present glutamine residues (Q) in gluten can be deamidated to glutamate residues (E) by tissue transglutaminase. Unfortunately this regiospecific deamidation of gliadin peptides further increases their immunogenic potential. Against this background we have been able to create an effective enzyme combination consisting of an *Aspergillus* derived proline specific endoprotease with an endoprotease to prevent the formation of proline rich toxic proline rich peptides. According to the present invention glutamate-specific endoproteases (EC3.4.21.19) can be used, for example those glutamate-specific endoproteases that are over secreted by a number of food-grade microorganisms such as *Bacillus* and *Streptomyces*. These enzymes

Page 28, line 3, after the Table, insert:

In the above Table, QLQFPQPQLPY is SEQ ID NO:7, QPFPQPQ is SEQ ID NO: 8, QPQQPQQSPFQQQRPF is SEQ ID NO:9, QQRPF is SEQ ID NO:10, QPPFSQQQQSPFSQ is SEQ ID NO:11, QSPFS is SEQ ID NO:12, PPFSQQ is SEQ ID NO:13.

Page 30, lines 11-29, please amend as follows:

Owing to a specific structural feature, prolyl oligopeptidases belonging to the S9 family cannot digest peptides larger than 30 amino acids. This limitation is an obvious disadvantage for an enzyme, which is meant to hydrolyse as quickly and as efficiently as possible all potential proline rich toxic proline rich peptides. To see if the *A. niger* derived proline specific endoprotease exhibits the same limitations with respect to the size of the substrate molecule, we have incubated the chromatographically purified prolyl endopeptidase from *A. niger* with a small synthetic peptide and with the large ovalbumine molecule and have analysed the hydrolysis products formed by SDS-PAGE. The synthetic peptide used was a 27-mer of the sequence NH2-FRASDNRVIDPGKVETLTIRRLHIPR-COOH (SEQ ID NO:14) and was a gift of the Pepscan company (Lelystad, The Netherlands). As shown by its amino acid sequence, this peptide contains 2 proline residues, one in the middle and one near the very end of the peptide. The intact ovalbumine molecule (Pierce Imject, vials containing 20mg freeze dried material) consists of 385 amino acids with a molecular weight of 42 750 Da. This molecule contains 14 proline residues one of which is located at the ultimate C-terminal end of the molecule and cannot be cleaved by a proline specific endoprotease.

Ovalbumin and the oligopeptide were separately incubated at 50°C with the purified *A. niger* derived proline specific endoprotease. At several time intervals samples were taken which were then analysed using SDS-PAGE.

Page 32, lines 26-32, please amend as follows:

Apart from the BCM-7 sequence Tyr-Pro-Phe-Pro-Gly-Pro-Ile (SEQ ID NO:15) at amino acid positions 60-66 of the beta-casein molecule, the smaller fragments like Tyr-Pro-Phe-Pro (beta-casomorphin (1-4)) (SEQ ID NO:16) and Tyr-Pro-Phe-Pro-Gly (beta-casomorphin (1-5)) (SEQ ID NO:17) at amino acid positions 60-63 and 60- 64 respectively as well as all larger peptides up to a chain 30 length of 11 amino acids (at amino acid positions 60-70) display at least some degree of opioid activity. The tripeptide Tyr-Pro-Phe at position 60-62 has been reported to have no opioid activity.

Page 33, line 7, after Table 1, insert the following:

In Table 1, the sequence identification numbers of the listed sequences beginning with YPFPGPI and extending to LVYPFGPIPNL are SEQ ID NO:18 to SEQ ID NO:31, respectively.

Page 33, lines 15-19, please amend as follows:

-The exact beta-casomorphine sequences i.e. YPFPGPI (SEQ ID NO:18) and derivatives are not present in (A2) beta-casein treated with either Alcalase or the combination of Alcalase plus the proline specific endoprotease from *A. niger*.

-However, two peptides containing the beta-casomorphin sequence are present in the Alcalase-treated sample i.e. LVYPFPGPIP (SEQ ID NO:29) and VYPFPGPIP (SEQ ID NO:26).

Page 34, line 3, after Table 2, insert the following:

In Table 2, the sequence identification numbers of the listed sequences beginning with YPFPGPI and extending to LVYPFPGPIPNSL are SEQ ID NO:18 to SEQ ID NO:31, respectively.

Page 34, lines 15 and 16, please amend as follows:

Peptides formed upon the incubation of the Alcalase formed peptide VYPFPGPIP (SEQ ID NO:26) with the proline specific endoprotease from *A. niger*.

Page 34, lines 18-23, please amend as follows:

As shown in Example 4, the hydrolysis of A2 beta-casein with a combination of Alcalase and the proline specific endoprotease from *A. niger* effectively removes all potential beta-casomorphin sequences. However, the complexity of the peptides generated did not allow us to establish at which position the *Aspergillus* derived enzyme cleaves the Alcalase formed peptide VYPFPGPIP (SEQ ID NO:26). To that end a peptide with this

Page 35, lines 2-33, please amend as follows:

Treatment of the 10-mer VYPFPGIPN (SEQ ID NO:26) (M= 1099.5) with 1 unit/g of protein already resulted in total breakdown of the 10-mer into several peptides. The intensity of the protonated molecule, at m/z 1100.5, drops 3 orders of magnitude. Treatment of the 10 mer with 10 units/g did not result in further decrease of the intensity of the protonated molecule and also no other peptide masses were found. Upon enzymatic treatment with 1 unit/g 4 peptides were formed, with VYP (M=377.2), characterized by m/z 378.2 as the most abundant (almost 98 %; see Table 3). All four peptides were analyzed in LC/MS/MS mode and found to be correct, based on the criteria described above. Table 3: Protonated peptide masses analyzed in LC/MS and LC/MS/MS mode of the 10-mer VYPFPGIPN (SEQ ID NO:26) M=1099.5. The second column presents the m/z values of the protonated molecules, the third column the intensity of the protonated molecules observed in LC/MS mode, the fourth column the percentage based on peak area of the protonated molecule and the fifth column the position of the peptides found in the total amino acid sequence of the 10-mer. It should be emphasized that using peak areas of

Page 36, lines 4 and 5, please amend as follows:

Table 3: Peptides formed upon the incubation of the BCM-7 related 10-mer VYPFPGIPN (SEQ ID NO:26) with the *A. niger* derived proline specific endoprotease.

Page 36, line 6, after Table 3, insert the following:

In Table 3, VYPF is SEQ ID NO:32, VYFPF is SEQ ID NO: 33, VYFPFPGP is
SEQ ID NO:34.

Page 36, lines 24-26, please amend as follows:

Peptides formed upon the incubation of the gliadin derived 33-mer
LQLQPFPPQLPYPQPQLPYPQPQLPYPQPQPF (SEQ ID NO:35) with the proline
specific endoprotease from *A. niger*.

Page 37, lines 1-10, please amend as follows:

Treatment of the gastric and pancreatic juices resistant gliadin derived 33-mer
LQLQPFPPQLPYPQPQLPYPQPQLPYPQPQPF (SEQ ID NO:35) (M=3911) as
described by Shan *et al* (Science, Vol 297, 27 September 2002) with Alcalase at either
pH 8 or pH 5 did not result in any cleavage of the molecule. However, similar to the
situation with the beta casein derived 10-mer, incubation with 1 unit of the proline
specific endoprotease from *A. niger* at pH 5 resulted in total breakdown of the molecule
into several peptides. The intensity of the triple protonated 33-mer at m/z 1304.4, drops
3 orders of magnitude. No further decrease of the intensity of the protonated molecule
and also no other peptide masses were observed upon treatment of the 33-mer with 10
enzyme units per gram of protein.

Page 37, lines 11-20, please amend as follows:

After enzymatic treatment about 6 main peptides and several minor peptides were formed, with a peptide characterized by m/z 565.2 as the most abundant. All 6 peptides were analyzed in LC/MS/MS mode and they all were found to contain proline at the C-terminus, confirming the enzyme's specificity. The major peptide formed is characterized by m/z 565.2 (sequence ..QLP in table 4). Although the C-terminal sequence "LP" could be unambiguously demonstrated for this peptide, the identified mass can theoretically not be formed by endoproteolytic degradation of the 33-mer so that there remains some uncertainty regarding the exact N-terminal composition of the peptide. Most probably m/z 565.2 is the N-pyroglutamyl variant of QPQLP (SEQ ID NO:36) ($M=581.3$), although this was not further investigated.

Page 37, lines 22 and 23, please amend as follows:

Appearance of QPQLP (SEQ ID NO:36): LQLQPFP QPQLP YP QPQLP YP
QPQLP YPQPQPF (SEQ ID NO:35)

Appearance of QPQLPYP (SEQ ID NO:37): LQLQPFP
QPQLPYPQPQLPYPQPQLPYP QPQPF (SEQ ID NO:35).

Page 37, lines 24-30, please amend as follows:

The LC/MS/MS spectrum of the peptide with m/z 679 could be elucidated to be PQPQLP (SEQ ID NO:39). Despite the fact that the nature of the peptide with m/z 565.2 was not fully understood, the data obtained clearly demonstrate the preferential cleavage of the proline specific endoprotease from *Aspergillus* at the C-terminal side of the proline residues at positions 12, 19 and 26 (i.e. exclusively between the proline and the tyrosine residue) of this particular 33-mer. This cleavage pattern is not influenced by using higher enzyme/substrate ratios. In table 4 all relevant information is summarized.

Page 38, lines 4-6, please amend as follows:

Table 4: Peptides formed upon incubation of the gliadin derived 33-mer LQLQPFQPQLPYPQPQLPYPQPQLPYPQPQPF (SEQ ID NO:35) with the proline specific endoprotease from *A. niger*.

Page 38, line 9 after Table 4, insert the following:

In Table 4, YPQPQLP is SEQ ID NO:37, QPQP is SEQ ID NO:38, PQPQLP is SEQ ID NO:39.

Page 38, lines 10-23, please amend as follows:

Cleavage of the 33-mer, claimed to be a major epitope in celiac patients, cannot be accomplished by gastric or pancreatic juices or by incubation with the aggressive broad spectrum protease Alcalase, neither under alkaline nor under acid conditions. Nevertheless our results indicate an efficient cleavage by the proline specific *A. niger* derived endoprotease under acid conditions. The latter cleavage takes place exclusively between the proline and the tyrosine residues of the molecule and generates 99.5% of peptides with no more than 6 amino acid residues long. So, despite its high efficacy towards proline rich peptides under acid conditions, even the *Aspergillus* derived enzyme leaves at least 0.5% of a heptamer with the amino acid sequence YPQPQLP (SEQ ID NO:40). As the sequence PYPQPQLPY (SEQ ID NO:41) is a known celiac patient-specific T cell epitope, this finding emphasizes once more that for suboptimal proline specific enzymes with near neutral pH optima such as the known proline specific oligopeptidases and the enzyme derived from *Flavobacterium meningosepticum*, a realistic *in vivo* application to prevent the formation of toxic peptides from gluten molecules will proof to be impossible.

Page 44, line 4 after Table 6, insert the following;

In Table 6, the sequence identification numbers for the listed sequences beginning with PQPQLPYQPQLPY through to QPQPFQSQSEQSQPFQPF are SEQ ID NOS: 42 and 44-52, respectively.

Page 45, line 3 after Table 7, insert the following;

In Table 7, the sequence identification numbers for the listed sequences beginning with QQP//FVQQQQP//FVQ through to QP//FP//QP//QQPFPQSQ are SEQ ID NOS: 53-58, respectively.

After the specification and immediately prior to the claims, please insert the sequence listing pages submitted herewith.